# *ABA-Hypersensitive Germination3* Encodes a Protein Phosphatase 2C (AtPP2CA) That Strongly Regulates Abscisic Acid Signaling during Germination among Arabidopsis Protein Phosphatase 2Cs[1][W]

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The phytohormone abscisic acid (ABA) regulates physiologically important developmental processes and stress responses. Previously, we reported on Arabidopsis (*Arabidopsis thaliana*) L. Heynh. *ahg* mutants, which are hypersensitive to ABA during germination and early growth. Among them, *ABA-hypersensitive germination3* (*ahg3*) showed the strongest ABA hypersensitivity. In this study, we found that the *AHG3* gene is identical to *AtPP2CA*, which encodes a protein phosphatase 2C (PP2C). Although AtPP2CA has been reported to be involved in the ABA response on the basis of results obtained by reverse-genetics approaches, its physiological relevance in the ABA response has not been clarified yet. We demonstrate in vitro and in vivo that the *ahg3-1* missense mutation causes the loss of PP2C activity, providing concrete confirmation that this PP2C functions as a negative regulator in ABA signaling. Furthermore, we compared the effects of disruption mutations of eight structurally related PP2C genes of Arabidopsis, including *ABI1*, *ABI2*, *HAB1*, and *HAB2*, and found that the disruptant mutant of *AHG3/AtPP2CA* had the strongest ABA hypersensitivity during germination, but it did not display any significant phenotypes in adult plants. Northern-blot analysis clearly showed that *AHG3/AtPP2CA* is the most active among those PP2C genes in seeds. These results suggest that AHG3/AtPP2CA plays a major role among PP2Cs in the ABA response in seeds and that the functions of those PP2Cs overlap, but their unique tissue- or development-specific expression confers distinct and indispensable physiological functions in the ABA response.

The plant hormone abscisic acid (ABA) mediates various aspects of developmental or physiological processes of plants, such as seed maturation, dormancy, germination, stomatal regulation, and sensitivity to other plant hormones such as ethylene and jasmonic acid. ABA also plays a major role in adaptation to abiotic environmental stresses, such as drought, salt, and cold (Leung and Giraudat, 1998).

Considerable efforts to understand the ABA response mechanisms, using forward- and reverse-genetics approaches mainly with Arabidopsis (*Arabidopsis thaliana*) L. Heynh., have identified a number of components implicated in the ABA-signaling pathway (for review, see Finkelstein et al., 2002). *ABI3*, *ABI4*, and *ABI5* encode different types of transcription factors that regulate the expression of ABA-responsive genes in seeds (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000). ABI5 seems to interact directly with ABI3 (Nakamura et al., 2001) and to be negatively regulated by a ubiquitin-dependent protein degradation system involving AFP, an ABI5-interacting protein (Lopez-Molina et al., 2003). ERA1, a $\beta$-subunit of farnesyl transferase, is thought to act as a negative regulator of ABA signaling (Cutler et al., 1996) and to modulate the expression of *ABI3* (Brady et al., 2003). *HYL1*, *ABH1*, and *SAD1*, isolated in studies of Arabidopsis ABA-hypersensitive mutants, encode a double-stranded RNA-binding protein, an mRNA cap-binding protein, and an Sm-like snRNP, respectively. These proteins are thought to be involved in mRNA processing of some genes implicated in the ABA response (Lu and Fedoroff, 2000; Hugouvieux et al., 2001; Xiong

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et al., 2001). Recently, another ABA-hypersensitive locus *AHG2* was shown to encode a polyA-specific ribonuclease, indicating the involvement of mRNA degradation in ABA and stress responses (Nishimura et al., 2005). Analysis of another ABA-hypersensitive mutant, *rpn10*, suggests the involvement of the 26S proteasome system in ABA signaling (Smalle et al., 2003). A G-protein-coupled receptor, GCR1, and a G-protein $\alpha$-subunit, GPA1, are thought to be involved in ABA-mediated stomatal closure (Wang et al., 2001; Pandey and Assmann, 2004).

Protein phosphorylation and dephosphorylation are involved in ABA signaling. AAPK, an Snf-1-related protein kinase 3 (SnRK3) of *Vicia faba* and OST1/SnRK2E of Arabidopsis are required in the ABA response of guard cells (Li et al., 2000; Mustilli et al., 2002; Yoshida et al., 2002). PKABA1, a member of the Triticum SnRK2 family, is thought to function in ABA signaling in seeds (Johnson et al., 2002). Analysis of the *rcn1* mutant suggests that a protein phosphatase 2A functions as a positive signal transducer of early ABA signaling (Kwak et al., 2002). In addition, several protein phosphatase 2Cs (PP2Cs) are deeply involved in ABA signaling (Rodriguez, 1998; Schweighofer et al., 2004). *ABI1* and *ABI2* encode homologous PP2Cs that had been identified in studies of two dominant ABA-insensitive mutants, *abi1-1* and *abi2-1* (Leung et al., 1994, 1997; Meyer et al., 1994). Intragenic suppressors of *abi1-1* and *abi2-1* were isolated and shown to be ABA hypersensitive, indicating their functions as negative regulators of the ABA response (Gosti et al., 1999; Merlot et al., 2001). Results consistent with this idea were obtained in studies of Arabidopsis *HAB1/AtP2C-HA* and *Fagus sylvatica FsPP2C1* (Gonzalez-Garcia et al., 2003; Leonhardt et al., 2004; Saez et al., 2004). AtPP2CA, another Arabidopsis PP2C, was shown to block ABA signal transduction when transiently expressed in maize (*Zea mays*) mesophyll protoplasts (Sheen, 1998). Downregulation of *AtPP2CA* by an antisense gene accelerated plant development and led to freezing tolerance (Tahtiharju and Palva, 2001). These results indicate the role of AtPP2CA as a negative regulator of the ABA response. However, the physiological relevance of AtPP2CA in the ABA response has not been clarified because of the lack of a loss-of-function mutant to date.

Here, we describe the study of a novel Arabidopsis ABA-hypersensitive mutant, *ABA-hypersensitive germination3* (*ahg3*), which has been isolated by screening with an ABA analog (Nishimura et al., 2004). *AHG3* is identical to *AtPP2CA* and *ahg3-1* is a type of loss-of-function mutation. We examined the ABA sensitivity in germinating seeds of T-DNA or Dissociation (*Ds*) transposon insertion mutants of ABA-implicated PP2Cs and found that the mutation of *AHG3/AtPP2CA* had the strongest effect. Our results confirm the presumption that AHG3/AtPP2CA functions as a negative regulator of the ABA-signaling pathway and suggest that AHG3/AtPP2CA has a major role in ABA signaling in seed germination and early growth.

## RESULTS

### Physiological Characterization of *ahg3-1*

*ahg3-1* is an ABA-hypersensitive mutant that germinates and grows poorly in the presence of ABA (Nishimura et al., 2004). To elucidate the function of AHG3 in the ABA response, we investigated the efficiency of radicle emergence and early growth of this mutant in the presence of various concentrations of ABA. *ahg3-1* plants showed a slight, but clear, reduction in radicle emergence in the presence of exogenous ABA (Fig. 1, A and C). By contrast, they showed strong growth retardation after germination (Fig. 1, B, D, and H). We examined the effect of seed stratification on the germination of *ahg3-1*. As shown in Figure 1, E and F, without stratification the *ahg3-1* seeds germinated and grew poorly. In contrast, stratification treatments dramatically improved those abilities.

To clarify whether the ABA hypersensitivity of *ahg3-1* is due to higher accumulation of ABA, we examined the endogenous ABA levels in seeds. Interestingly, the dry *ahg3-1* seeds accumulated 150% more endogenous ABA than the wild type. However, after stratification at 4°C for 4 d (the conditions we usually used), the endogenous ABA level of *ahg3-1* decreased to the same level as the wild type (Fig. 1G). These results suggest that the lower germination and postgermination growth efficiencies of *ahg3-1* without stratification are due, at least partially, to the higher accumulation of endogenous ABA. This idea is consistent with the effect of stratification (Fig. 1, E and F).

The *ahg3-1* plants grew slightly slower than wild-type plants on soil, but the final plant size was the same as the wild type. The bolting time was also slightly delayed; the wild-type plants started to bolt at 22 to 25 d after sowing, but *ahg3-1* plants needed 2 to 3 d longer. However, the numbers of rosette leaves when bolting started were almost the same in wild-type and *ahg3-1* plants (wild type, 12.5 $\pm$ 0.85; *ahg3-1*, 12.7 $\pm$ 0.82; $n = 10$), suggesting that the delay in bolting of *ahg3-1* is due not to abnormal flowering control but to slower growth. These results suggest that growth retardation is due not to altered ABA sensitivity in later growth stages but presumably to slower germination of *agh3-1* (Fig. 1, E and F). We examined the ABA sensitivity of rosette plants. Seven-day-old seedlings were placed on an ABA-containing plate and grown for 10 d. The *ahg3-1* plants grew identically to the wild type (data not shown), implying that *ahg3-1* does not have a detectable ABA-hypersensitive phenotype in the adult stage under our experimental conditions. In the previous study, we examined the expression of ABA- and stress-inducible genes—*RD29A*, *RD29B*, *P5CS*, and *RAB18*—by RNA gel-blot analysis and found no significant changes in *ahg3-1* (Nishimura et al., 2004). In this study, we examined those in more detail and obtained the same results (Supplemental Fig. 1). These results suggest that *ahg3-1*

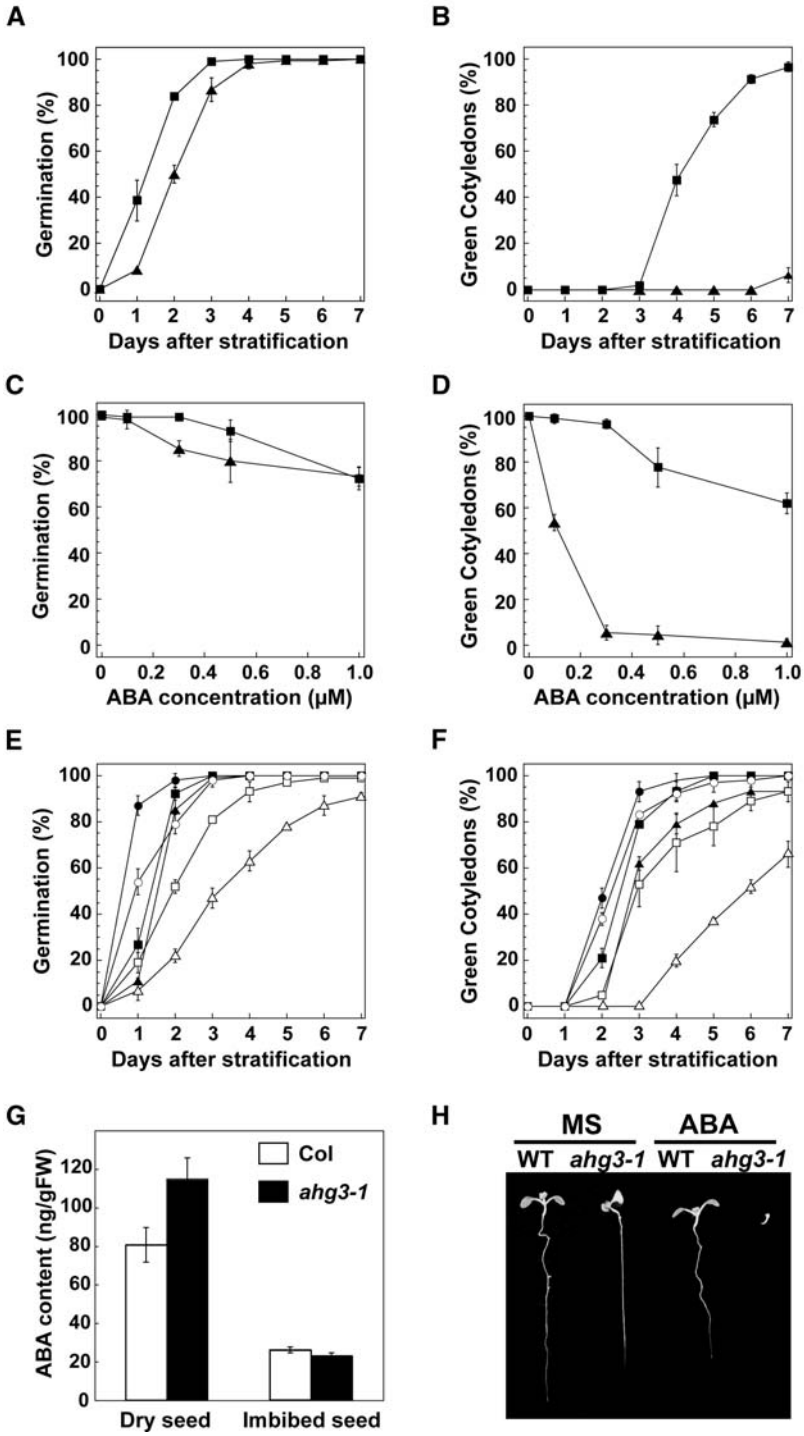



**Figure 1.** ABA-hypersensitive phenotype of *ahg3-1*. A and C, Germination efficiencies (radicle emergence); B and D, postgermination growth efficiencies (green cotyledons) of wild-type (black squares) and *ahg3-1* (black triangles) seeds in the presence of 0.3 $\mu$M ABA for 7 d after stratification (A and B) or seeds in the presence of various concentrations of ABA at 3 d (C) or 7 d (D) after stratification. E and F, Dormancy of wild-type (black symbols) and *ahg3-1* (white symbols) seeds; triangles, squares, and circles indicate seeds imbibed for 0, 2, and 4 d at 4°C, respectively. In A to F, averages of three independent experiments are shown with SDs. Approximately 50 seeds were used in each experiment. G, Endogenous ABA content of dry and imbibed seeds (4°C for 4 d) of wild type (white bars) or *ahg3-1* (black bars). Averages of three independent experiments are shown with SDs. H, Wild-type and *ahg3-1* seedlings grown on MS plates with or without 0.3 $\mu$M ABA for 7 d after stratification.

does not affect ABA sensitivity so strongly in adult plants under the imposed conditions.

### Double-Mutant Analysis

To characterize *ahg3-1* genetically, we performed epistatic analysis. *ahg3-1* plants were crossed with *abi1-1*, *abi2-1*, *abi3-1*, *abi4-1*, and *abi5-1* plants, and double-mutant lines were obtained from $F_2$ progeny. We examined the early growth efficiencies of those lines on Murashige and Skoog (MS) plates containing 0.3 or 3.0 $\mu$M ABA (Table I). The *ahg3-1abi1-1*, *ahg3-1abi4-1*, and *ahg3-1abi5-1* double mutants showed less ABA resistance than the parental monogenic mutant, suggesting additive effects between *ahg3-1* and these *abi* mutations. By contrast, *ahg3-1* had little or no effect on

**Table I.** *Early growth efficiency of single and double mutants on ABA plates*

Approximately 50 seeds were used. Values show percent of seedlings with green cotyledons after a 10-d incubation. Averages of three independent experiments are shown with SDs. Col, Columbia; Ws, Wassilewskija.

| Genotype | MS | 0.3 μM ABA | 3.0 μM ABA |
|---|---|---|---|
| Col | 100 | 89.7 ± 6.4 | 6.22 ± 5.6 |
| L*er* | 100 | 90.5 ± 3.4 | 2.01 ± 2.2 |
| Ws | 100 | 85.7 ± 4.4 | 0.53 ± 0.9 |
| *ahg3-1* | 100 | 8.07 ± 7.2 | 0 |
| *abi1-1* | 100 | 98.7 ± 2.3 | 94.3 ± 0.3 |
| *abi2-1* | 100 | 95.6 ± 7.7 | 75.6 ± 5.4 |
| *abi3-1* | 87.2 ± 2.5 | 84.3 ± 5.4 | 82.9 ± 3.1 |
| *abi4-1* | 100 | 100 | 83.4 ± 1.1 |
| *abi5-1* | 100 | 95.9 ± 1.7 | 94.1 ± 1.2 |
| *ahg3-1abi1-1* | 100 | 79.7 ± 8.2 | 57.0 ± 5.3 |
| *ahg3-1abi2-1* | 99.4 ± 1.1 | 96.3 ± 4.6 | 89.8 ± 3.6 |
| *ahg3-1abi3-1* | 91.9 ± 7.1 | 97.6 ± 0.2 | 98.9 ± 1.8 |
| *ahg3-1abi4-1* | 100 | 97.6 ± 2.1 | 51.0 ± 1.8 |
| *ahg3-1abi5-1* | 100 | 96.1 ± 1.3 | 60.2 ± 3.1 |

the ABA sensitivity of *abi2-1* and *abi3-1*. The germination efficiency of *abi2-1* was reduced by 3.0 μM ABA. The *ahg3-1* mutation did not seem to enhance this ABA effect. Even 3.0 μM ABA had only a subtle effect on the germination efficiencies of both *abi3-1* and *ahg3-1abi3-1*. We obtained almost the same results using independent double-mutant lines, suggesting that the effect of the ecotype background was negligible (data not shown). These results might indicate that *AHG3* functions at or upstream of *ABI2* and *ABI3* if the ABA-signaling pathway is linear.

### *AHG3* Encodes a PP2C, AtPP2CA

We identified the *AHG3* gene by map-based cloning. The *AHG3* locus was mapped near the marker nga162 on chromosome 3 (Nishimura et al., 2004). We analyzed 1,746 chromosomes of $F_2$ plants obtained from an *ahg3-1* × Landsberg *erecta* (L*er*) test cross and narrowed the *AHG3* locus to a region spanning about 70 kb (Fig. 2A). By determining the nucleotide sequence of this region, we found a base conversion from G to A in the third exon of *At3g11410* (Arabidopsis Genome Initiative, 2000), which encodes a PP2C, AtPP2CA (Kuromori and Yamamoto, 1994). This mutation causes the amino acid change of Gly-287 to Glu in the predicted gene product. Interestingly, the *ahg3-1* mutation occurred at the corresponding amino acid residue of the *abi1-1R6* mutation site, one of the intragenic suppressor mutants of *abi1-1* (Fig. 2B; Gosti et al., 1999). We obtained a T-DNA insertion allele of *AHG3* from the SALK T-DNA lines (SALK_028132; Alonso et al., 2003). The T-DNA was inserted in the end of the second exon; we named this line *ahg3-2* (Fig. 2A). Northern-blot analysis of *ahg3-2* failed to detect the correct size of *AHG3* mRNAs, suggesting that *ahg3-2* is a null allele (Fig. 2C). *ahg3-2* showed ABA-hypersensitive phenotypes in germination and early growth as strong as those of *ahg3-1* (Fig. 2D).

From these results, we presumed that ABA hypersensitivity of *ahg3-1* was due to a defect of the PP2C enzymatic activity of AHG3/AtPP2CA. To confirm this presumption, we performed an in vitro PP2C assay using $^{32}$P-labeled casein as a substrate in the presence of okadaic acid, an inhibitor of other types of phosphatases, PP1 and PP2A (Mackintosh and Moorhead, 1999). Recombinant glutathione *S*-transferase (GST) fusion proteins of AHG3, ahg3-1, and ahg3$^{G145D}$, which carries an *abi1-1*-type mutation (Sheen, 1998; Fig. 2E), were expressed in *Escherichia coli* and affinity purified (Fig. 2F). The results are summarized in Figure 2G. Protein ahg3-1 showed a PP2C activity less than one-hundredth of the AHG3 activity and similar to that of ahg3$^{G145D}$. This result suggests that the *ahg3-1* mutation causes the defect in PP2C activity.

To confirm that *At3g11410* is *AHG3*, we generated a transgenic *ahg3-1* plant possessing a wild-type *At3g11410* gene. Transgenic plants showed normal early growth in the presence of ABA (Fig. 2H). From these results, we concluded that *At3g11410* is *AHG3*. Taken together, these results strongly suggest that the defect in AHG3/AtPP2CA caused the ABA-hypersensitive phenotype and that AHG3/AtPP2CA is indeed a negative regulator of ABA signaling.

### *ahg3-2* Showed the Strongest ABA Hypersensitivity among T-DNA Insertion Mutants of ABA-Related PP2Cs

At least four Arabidopsis PP2C genes, *ABI1*, *ABI2*, *HAB1/AtP2C-HA*, and *AHG3/AtPP2CA*, have been shown to be ABA inducible (Rodriguez, 1998) and are thought to function in the ABA-signaling pathway. So far, there is very little information about the redundant or distinct roles of their proteins. It is of interest to clarify whether our isolation of *ahg3-1* as an ABA-hypersensitive mutant reflects a specific function of AHG3/AtPP2CA or happened just by chance. We obtained T-DNA or *Ds* transposon insertion mutants of PP2C genes closely related to *AHG3/AtPP2CA* (Alonso et al., 2003; Kuromori et al., 2004; Ito et al., 2005): *ABI1* (*abi1td*, SALK_076309; *abi1ds*, 13-4144-1); *ABI2* (*abi2td*, SALK_015166); *HAB1/AtP2C-HA* (*hab1-1*, SALK_002104; Leonhardt et al., 2004; Saez et al., 2004); *HAB2* (*hab2ds*, 15-1860-1); *At2g29380* (*at2g29380td*, SALK_033011); *At1g07430* (*at1g07430ds*, 53-2196-1); and *At5g59220* (*at5g59220ds*, 53-2870-1; Fig. 3). The T-DNA or *Ds* insertion sites of *abi1td*, *abi1ds*, *hab1-1*, *at1g07430ds*, and *at5g59220ds* are located in the coding regions, indicating that these are null mutations. The insertion sites of *hab2ds* and *at2g29380td* are located in an intron and the 5′-untranslated region, respectively. The expression of these genes was undetectable by reverse transcription (RT)-PCR, indicating that these also are null mutations (see Supplemental Fig. 2). The T-DNA insertion site of *abi2td* is the C terminus of the *ABI2*-coding region. We could not conclude whether this mutation was null or not. However, the phenotype

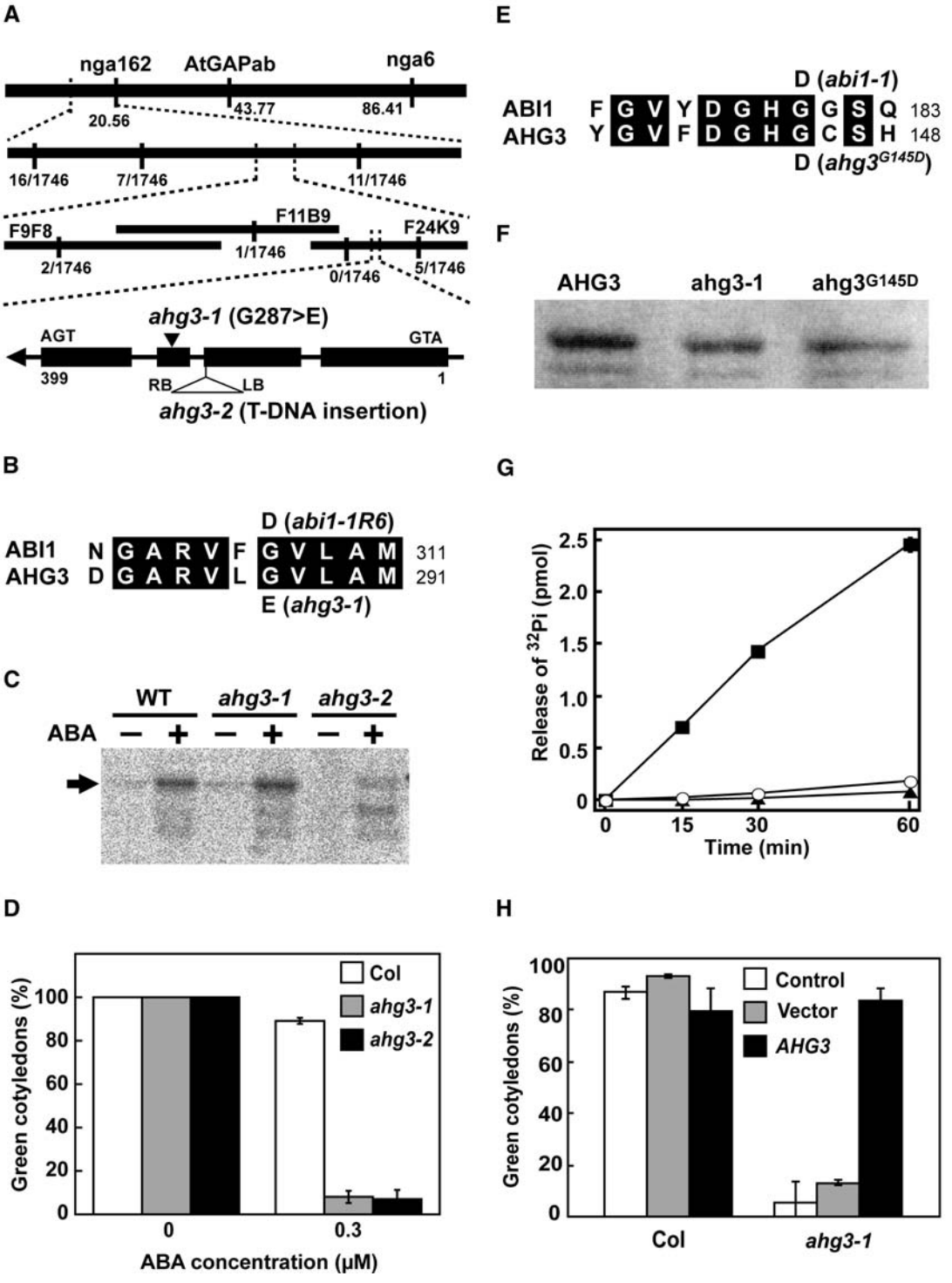



**Figure 2.** Identification of *AHG3* by map-based gene cloning. A, Schematic representations of mapping and structure of *AHG3*. The exon-intron organization of *AHG3* is shown. Mutation sites of *ahg3-1* and *ahg3-2* (T-DNA insertion) are indicated. B, Comparison of the amino acid changes caused by *ahg3-1* and *abi1-1R6*. White letters indicate conserved amino acid residues. C, RNA gel-blot analysis of *AHG3* in wild type, *ahg3-1*, and *ahg3-2*. D, ABA hypersensitivity of *ahg3-2*. Seeds were sown on a MS plate containing 0 or 0.3 $\mu$M ABA and grown for 7 d. The percentages of seedlings with green cotyledons are shown. Averages of three independent experiments are shown with SDs. Approximately 50 seeds were used in each experiment. E, Amino acid sequence of the *ahg3*$^{G145D}$ artificial mutation similar to *abi1-1*. White letters indicate conserved amino acid residues. F, Coomassie Blue staining of GST-fusion proteins used in the in vitro phosphatase assay. Each lane contains approximately 1 $\mu$g of protein. G, In vitro phosphatase activity of recombinant proteins. GST-AHG3 (20 ng; black squares), GST-ahg3-1 (100 ng; black triangles), and GST-ahg3$^{G145D}$ (100 ng; white circles) were incubated with $^{32}$P-labeled casein in the presence of 2 $\mu$M okadaic

(see below) is stronger than that of the *abi2-1* revertant mutant reported previously (Merlot et al., 2001). Therefore, it is plausible that the *abi2td* mutation also reduced the function of ABI2 to a very low level. The homozygosity of the T-DNA or *Ds* insertion was confirmed by examining the genome structure by a PCR-based method.

To see the effect of insertional mutations of these PP2Cs on the ABA response during germination, we examined the growth efficiencies of mutants in the presence of ABA. We could not observe any significant effects of the insertion mutations of *At2g29380*, *At1g07430*, or *At5g59220*. By contrast, the *ahg3-2*, *abi1td*, *abi2td*, *hab1-1*, and *hab2ds* insertion mutations seemed to enhance ABA sensitivity, consistent with previous results. Interestingly, *ahg3-2* had the strongest effect (Fig. 4, A and B; data not shown). To measure ABA sensitivity more objectively, we measured the chlorophyll content of 10-d-old seedlings (Fig. 4C). The mutants of *At2g29380*, *At1g07430*, and *At5g59220* accumulated chlorophyll normally, while the others accumulated less. Among those, the strongest effect of *ahg3-2* was again obvious. These results suggest that AHG3/AtPP2CA plays a major role as a negative regulator of ABA signaling during seed germination and early growth, although ABI1, ABI2, HAB1, and HAB2 are also involved.

### AHG3/AtPP2CA Is Strongly Expressed in Seeds

The results described above raised the question of what determines the major role in seeds of AHG3/AtPP2CA among related PP2Cs. The total amino acid sequence similarity cannot account for it, because the insertion mutations of *At2g29380*, *At1g07430*, and *At5g59220*, which are closely related to *AHG3/AtPP2CA* (Fig. 5B), did not have any effect. According to published DNA microarray experiment data (Nakabayashi et al., 2005; AtGenExpress), the mRNA levels of these PP2C genes are considerably different between dry and imbibed seeds. Therefore, it might be possible that the differences in the expression of these genes explain the different contributions of these PP2Cs to ABA signaling in seeds. We performed RNA gel-blot analyses to investigate the mRNA levels of *AHG3/AtPP2CA* and several other PP2C genes in seeds. Gene-specific probes were designed from dissimilar N-terminal regions, and the signal strength of each probe was validated by genome dot-blot hybridization (see Supplemental Fig. 3). As expected, in dry seeds, the expression of *AHG3/AtPP2CA* was much stronger than those of *ABI1*, *HAB1*, and *HAB2* (Fig. 5A, lane a). The mRNA level of *AHG3/AtPP2CA* did not seem to change during stratification and germination (Fig. 5A, lanes b and c; Supplemental Fig. 4). Using the same probes, we examined the mRNA levels in adult plants. These mRNA levels were up-regulated by ABA treatment and seemed similar among the PP2C genes. These results show that the expression level of *AHG3/AtPP2CA* is significantly higher in seeds, but the same as other PP2C genes in adult plants.

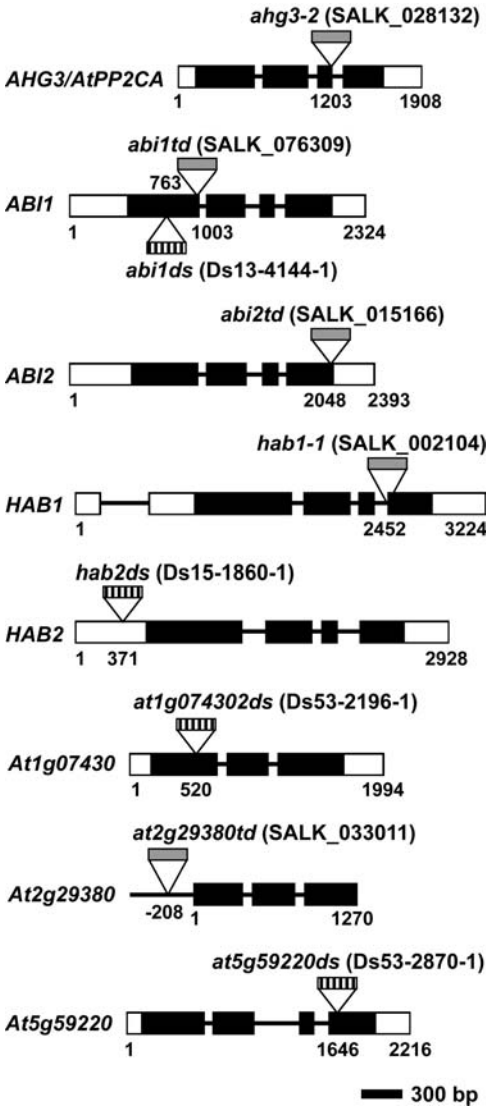



**Figure 3.** T-DNA or *Ds* insertional mutations of PP2C genes. Schematic representation of the T-DNA or *Ds* insertion site of PP2C mutants used in this study. Gray box and striped box indicate T-DNA and *Ds* transoposon, respectively. White box, black box, and horizontal thick line indicate 5′- or 3′-untranslated region, exon, and intron, respectively. Number indicates the nucleotide position from the cDNA start point. There is no cDNA reported for *At2g29380*.

**Figure 2.** (*Continued.*)
acid. PP2C activity is expressed in picomoles of released $^{32}$Pi. Values shown are the means of duplicate assays. The error bar is not shown if it is smaller than the symbol size. H, Complementation analysis of *AHG3*. Seeds of transgenic *ahg3-1* plants possessing the genomic *AHG3* clone or vector-control plants were germinated and grown in the presence of 0.3 $\mu$M ABA for 7 d. Averages of three independent experiments are shown with SDS. Approximately 50 seeds were used in each experiment.

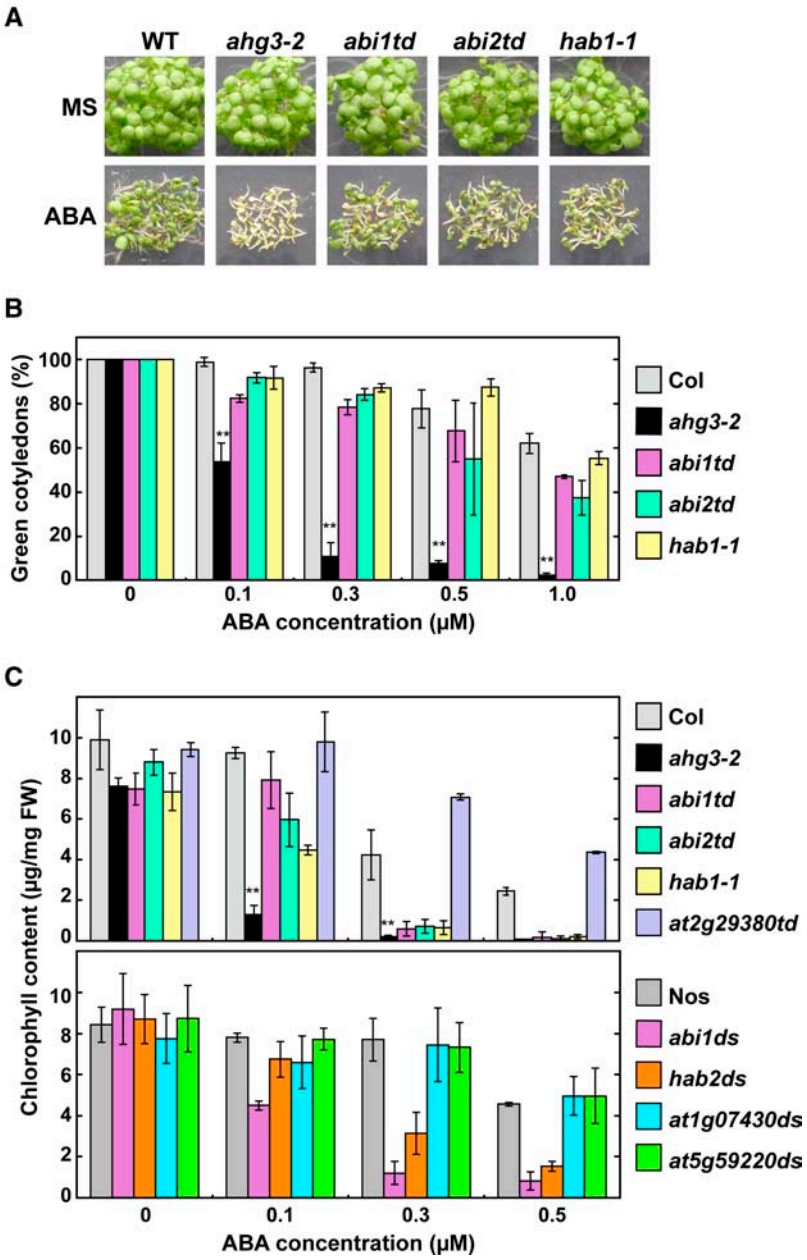
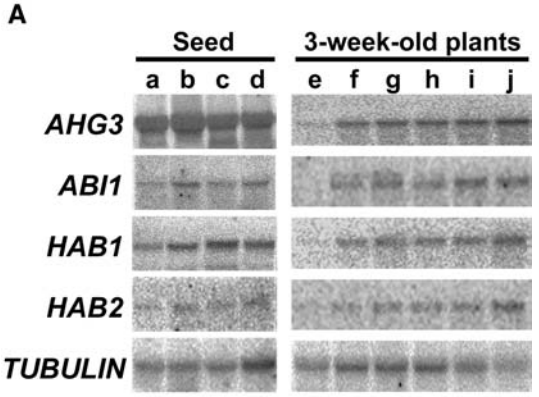



**Figure 4.** ABA sensitivity of *ahg3-2* was stronger among ABA-related PP2C insertion mutants. A, Seedlings of wild-type plants and PP2C insertion mutants germinated in the presence of 0 or 0.3 $\mu$M ABA. Photographs were taken after a 7-d incubation. B, Postgermination growth efficiency of wild-type plants and PP2C insertion mutants. About 50 seeds were sown on MS plates containing various concentrations of ABA and incubated for 7 d. C, Chlorophyll content of T-DNA insertion lines (top) and *Ds* transposon insertion lines (bottom). Seeds were sown on MS plates containing various concentrations of ABA and incubated for 10 d. In B and C, averages of three independent experiments are shown with SDs. Approximately 50 seeds were used in each experiment.

### The $P_{35S}$:*AHG3/AtPP2CA* Fusion Gene and the *ahg3*$^{G145D}$ Gene Confer ABA Insensitivity

If AHG3/AtPP2CA is a negative regulator of the ABA response in seeds, overexpression of AHG3/AtPP2CA should confer ABA insensitivity. To evaluate this idea, we constructed transgenic plants possessing the $P_{35S}$:*AHG3/AtPP2CA* fusion gene. $T_2$ seeds obtained from resultant transgenic lines expressing the transgene showed considerable ABA insensitivity (Fig. 6A), confirming that AHG3/AtPP2CA functions as a negative regulator.

Two dominant PP2C mutations, *abi1-1* and *abi2-1*, have the same amino acid substitution. The amino acid change causes the defect of PP2C activity (this study; Gosti et al., 1999; Merlot et al., 2001). Therefore, *abi1-1* and *abi2-1* mutations are thought to be dominant-negative mutations, although their molecular mechanisms have not been described yet. The amino acid sequence around the mutation site is highly conserved among PP2Cs, including AHG3/AtPP2CA. A recent study showed that the sequence around this mutation site in ABI2 was required for the direct interaction with SOS2 protein kinase (Ohta et al., 2003). Therefore, presumably, this conserved amino acid sequence has an important function. To establish whether or not the same type of mutation in AHG3/AtPP2CA causes ABA insensitivity as well, we introduced to the wild-type plants a genomic DNA fragment containing *AHG3/AtPP2CA* with the same mutation as in *abi1-1*




B

| | ABA sensitivity[a] | mRNA level |
|---|---|---|
| ABI1 | + | + [b] |
| ABI2 | + | + [c] |
| HAB1 | + | + [b] |
| HAB2 | + | + [b] |
| At5g59220 | – | – [c] |
| At2g29380 | – | + [c] |
| At1g07430 | – | + [c] |
| AHG3 /AtPP2CA | ++ | ++ [b] |

**Figure 5.** Expression of PP2C genes in seeds. A, Northern-blot analysis of PP2C genes implicated in the ABA response in seeds and adult plants. Each lane contains approximately 10 $\mu$g of total RNA extracted from dry seeds (a), from seeds grown on MS plates for 1 d (b) and 3 d (c), from seeds grown on MS plates containing 1.0 $\mu$M ABA for 3 d (d), or from 3-week-old plants treated with 100 $\mu$M ABA for 0 (e), 1 (f), 2 (g), 5 (h), 10 (i), and 24 h (j), respectively, after preincubation in water for 2 h. The *β-TUBULIN* gene was used as a loading control. B, Relationship between overall structure, mRNA level in seeds, and effect of mutation on ABA sensitivity of PP2Cs. A phylogenetic tree was constructed using ClustalW. The ABA sensitivity of insertion mutants and mRNA levels in imbibed seeds are summarized. a and b, This study; c, according to the AtGenExpress microarray database.

and *abi2-1* at the corresponding site. The seeds obtained from transgenic plants exhibited clear ABA insensitivity, suggesting that the same mutation of *AHG3/AtPP2CA* as in *abi1-1* and *abi2-1* confers ABA insensitivity, and that AHG3/AtPP2CA has similar physiological and biochemical properties as ABI1 and ABI2.

## DISCUSSION

### Isolation of the *ahg3-1* Mutant

We characterized the Arabidopsis *ahg3-1* line, which displays strong ABA hypersensitivity during germination. We found that the *AHG3* gene is identical to *AtPP2CA*, which encodes a PP2C. *AtPP2CA* was originally cloned by transcomplementation using a fission yeast (*Schizosaccharomyces pombe*) mutant defective in meiosis (Kuromori and Yamamoto, 1994). Transient-assay experiments using maize mesophyll cells and studies with antisense genes implicated AtPP2CA in ABA signaling as a negative regulator (Sheen, 1998; Tahtiharju and Palva, 2001). However, its physiological relevance in the ABA response has been obscure. In this study, we clearly demonstrated that the defect in *AHG3/AtPP2CA* conferred ABA hypersensitivity. First, we showed that the recombinant ahg3-1 protein had undetectable PP2C activity in the in vitro assay. Second, we confirmed that *ahg3-2*, a null-type insertion mutant, displayed almost the same phenotype as *ahg3-1*, suggesting that *ahg3-1* mutation results in the defect in PP2C activity in vivo. Third, we constructed transgenic plants overexpressing this gene and found that they are ABA resistant during germination. Together, these results offer conclusive evidence that AHG3/AtPP2CA functions as a negative regulator of the ABA response.

In addition, analysis of *ahg3-1* offered further valuable information. So far, there are only two single missense mutations of PP2C, *abi1-1* and *abi2-1*, which are known to cause the dominant strong ABA insensitivity. It has been explained that *abi1-1* and *abi2-1* are dominant-negative mutants because the mutant

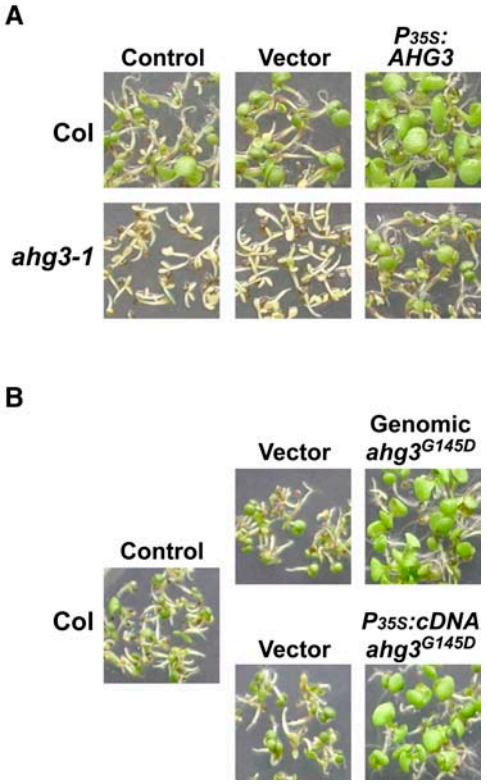



**Figure 6.** In planta effects of overexpression and *abi1-1*-type mutation of *AHG3*. Effect of overexpression of *AHG3/AtPP2CA* (A) or $ahg3^{G145D}$ (B) on ABA sensitivity during germination. Seeds were sown and grown on MS plates containing 0.3 $\mu$M ABA for 7 d.

proteins had significantly lower PP2C activity in in vitro assay (Gosti et al., 1999), although the molecular nature of these mutations is not yet fully understood. As shown above, the *ahg3-1* mutation alone causes the defect in PP2C function both in vivo and in vitro without affecting transcription (Fig. 2C). The *abi1-1*-type mutation in *ahg3*$^{G145D}$ allowed AHG3 to block the ABA response in seeds despite the absence of PP2C activity in the in vitro assay, indicating the similar physiological and biochemical properties of AHG3 and ABI1. If *abi1-1*, *abi2-1*, and *ahg3*$^{G145D}$ cause dominant-negative effects by expressing phosphatase-defective PP2C proteins, ahg3-1 would have a dominant-negative effect, but it does not. Therefore, it is more likely that a missense loss-of-function mutation of PP2C cannot confer a dominant-negative effect, and that *abi1-1* and *abi2-1* are, instead, gain-of-function mutations. This assumption can explain all the results except for that of the in vitro PP2C assay. That inconsistency might be due to the use of an artificial substrate. If this is the case, we should consider more carefully the results obtained using *abi1-1* and *abi2-1*, since gain-of-function mutations do not always reflect the original gene function.

**Redundant and Distinct Functions of PP2Cs**

Besides AHG3/AtPP2CA, three Arabidopsis PP2Cs (ABI1, ABI2, and HAB1) have been postulated to be negative regulators of ABA signaling (Sheen, 1998; Gosti et al., 1999; Merlot et al., 2001; Leonhardt et al., 2004; Saez et al., 2004). Merlot et al. (2001) reported that ABI1 and ABI2 contribute nearly 50% of the ABA-induced PP2C activity, indicating that other PP2Cs may also be involved in redundant ABA signaling. The functional redundancy of genes often becomes an obstacle to isolating mutants. So why could we easily isolate a loss-of-function allele of *AtPP2CA*? The most plausible explanation is that AHG3/AtPP2CA possesses major or unique roles in the ABA response in seed germination, so the ABA-hypersensitive phenotype of *ahg3-1* was not suppressed by other PP2Cs. To test this hypothesis, we performed a comparative analysis of eight PP2Cs belonging to the same group by using T-DNA or *Ds* insertion mutants. Most of these mutants showed ABA hypersensitivity during germination and early growth, as previously shown in *hab1-1* (Saez et al., 2004), but at different intensities. In our assay, among these disruptant mutants, *ahg3-2* showed the strongest ABA hypersensitivity in early growth (Fig. 4). This result indicates that AHG3/AtPP2CA is implicated more deeply in ABA signaling during germination and postgermination growth than other PP2Cs. We failed to detect any obvious phenotypes of *ahg3-1* in the adult stage under our experimental conditions. Although we cannot exclude the possibility that *ahg3-1* adult plants have some ABA-related phenotypes under certain conditions, it is more likely that the phenotype during germination is more significant.

So how does AHG3/AtPP2CA play a major role in seeds? *AHG3/AtPP2CA* was expressed more strongly in seeds than *ABI1*, *HAB1*, and *HAB2*, whose disruptive mutations affect ABA sensitivity during germination (Fig. 5A). We also checked the expression profiles of several PP2C genes in seeds in the AtGenExpress microarray database. Consistent with our results, according to this database, *AHG3/AtPP2CA* showed the strongest expression (Fig. 5B). The mechanisms that regulate PP2C are not understood at all. All plant PP2Cs examined so far are active in in vitro assays, implying that plant PP2Cs are intrinsically active. The fact that the expression of ABA-related PP2C genes is up-regulated by ABA leads to the possibility that PP2C action is regulated at the transcriptional level. We think that the higher expression of *AHG3/AtPP2CA* contributes to its predominant role in seeds. This idea is consistent with the lower expression of *AHG3/AtPP2CA* in the adult stage where we failed to detect any significant effects of *ahg3-1* under our experimental conditions. ABA-related PP2Cs have shown distinct tissue-specific expression patterns (Leung et al., 1997; Rodriguez et al., 1998; Tahtiharju and Palva, 2001; Saez et al., 2004), implying that such distinct expression patterns determine the physiological functions of each ABA-related PP2C.

Substrate specificity also might determine their specific function. Thus, it is important to identify the substrate so as to understand not only the mechanism of how PP2Cs regulate ABA signaling, but also their distinct roles. Yeast (*Saccharomyces cerevisiae*) two-hybrid screening allowed the isolation of potassium channel AKT2 as a substrate candidate for *AHG3/AtPP2CA* (Vranova et al., 2001; Cherel et al., 2002). Cherel et al. (2002) described the modulation of AKT2 activity by AHG3/AtPP2CA in animal cells and the overlapped tissue-specific expression of *AHG3/AtPP2CA* and *AKT2*. However, according to the AtGenExpress microarray database, the expression of *AKT2* is weaker in seeds than in other tissues, whereas that of *AHG3/AtPP2CA* is higher. In addition, we failed to detect any effects of the *Ds* insertion in an *AKT2* exon (15-5406-1) on ABA sensitivity during germination (T. Yoshida, T. Kuromori, and T. Hirayama, unpublished data). Therefore, it is likely that AHG3/AtPP2CA has other substrates in seeds.

The double-mutant analysis showed that *abi2-1*, but not *abi1-1*, can suppress *ahg3-1*. This result indicates that *abi2-1* has a stronger effect on AHG3/AtPP2CA function. This result seems consistent with the notion that ABI1 and ABI2 have different functions. However, ABI1 has no less important a role than ABI2 in ABA response in seeds because *abi1-1* and *abi2-1* have similar ABA insensitivity during germination, and *abi1-1* revertant mutants displayed stronger ABA hypersensitivity than *abi2-1* revertant mutants. Our results might reflect the stronger similarity between AHG3/AtPP2CA and ABI2 in substrate preference, spatial expression in seeds, or subcellular localization than that between AHG3/AtPP2CA and ABI1.

### Physiological Function of AHG3/AtPP2CA in Seeds

As described above, AHG3/AtPP2CA functions as a negative regulator of ABA signaling in seeds. Interestingly, the endogenous ABA level was increased in *ahg3-1*, but returned to a normal level after a 4-d stratification (Fig. 1G). Consistent with this result, unstratified *ahg3-1* seeds germinated poorly on normal media, but stratified seeds germinated as well as the wild-type seeds. These results indicate that *ahg3-1* seeds tend to accumulate more ABA during seed maturation, but the down-regulation of ABA after stratification seems normal. Most genes for ABA biosynthesis are up-regulated by ABA and are regulated by positive feedback (for review, see Xiong and Zhu, 2003). Thus, the reason why *ahg3-1* seeds accumulate higher levels of ABA might be the positive feedback of the ABA response. This higher accumulation of ABA in seeds contributes to ABA hypersensitivity in early growth by up-regulating or prolonging the expression of seed-specific genes (Nishimura et al., 2004). By contrast, after stratification, the endogenous ABA content of *ahg3-1* dropped to normal, while its mRNA level was unchanged (Fig. 5A; Supplemental Fig. 4), indicating that AHG3 is not involved in the mechanism of ABA down-regulation in seeds.

Analysis of double mutants demonstrated that *abi3-1* nearly suppressed *ahg3-1*, whereas *abi4-1* and *abi5-1* suppressed *ahg3-1* more partially, indicating that ABI3 plays an important role in the ABA response downstream of *AHG3/AtPP2CA*. It has been proposed that the transcriptional factors ABI3, ABI4, and ABI5 regulate ABA response in seeds. They recognize different cis-elements but function together (Soderman et al., 2000). The null mutants of *ABI3* cause severe defects in seed development and maturation (Nambara et al., 1992), whereas null mutations of *ABI4* and *ABI5* have less effect, indicating that ABI3 has the most important function among them in ABA-responsive gene expression in seed. Our results of double-mutant analysis are consistent with this idea. Recent studies showed that ABI4 is more implicated in sugar response, implying its distinct function (for review, see Gibson, 2004). ABI5 is thought to function with ABI3 (Nakamura et al., 2001). Arabidopsis has several genes for the ABI5-like protein ABF/AREB (Jakoby et al., 2002). Therefore, the reason for the partial suppression of *ahg3-1* by *abi5-1* might be the redundancy among these ABF/AREBs. Alternatively, it is possible that ABA signaling is not transduced by such a simple linear pathway. The additive effects of mutations may imply the higher complexity of the ABA-signaling network.

Transgenic Arabidopsis plants with lowered expression of *AHG3/AtPP2CA* did not display any phenotype of dehydration stress (Tahtiharju and Palva, 2001). Consistent with this result, we could not detect any significant difference in the drought response between *ahg3-1* and the wild type (data not shown). Similarly, loss-of-function mutations of *ABI1*, *ABI2*, and *hab1-1* did not affect transpiration rate, although *abi1-1*, *abi2-1*, and overexpression of *HAB1* caused higher transpiration rates (Murata et al., 2001; Saez et al., 2004). These results indicate that these PP2Cs, including AHG3/AtPP2CA, which are up-regulated by ABA in stomata (Leonhardt et al., 2004), are implicated in ABA-mediated stomatal closure, although their functions are redundant.

In summary, by mapping of *AHG3*, we identified a single missense loss-of-function allele of a PP2C gene. Our data confirm the function of AHG3/AtPP2CA as a negative regulator of the ABA response. Comparative analysis of insertion mutants of eight PP2C genes suggested that AHG3/AtPP2CA is implicated deeply in the ABA response in germinating seeds. Further analyses using mutants obtained in this study will provide additional insights into the role of AHG3/AtPP2CA and other PP2Cs in ABA signaling.

Kuhn et al. (2006) have conducted an independent screen for ABA-signaling components in Arabidopsis that is similar to our research. They have characterized the same protein phosphatase AtPP2CA showing ABA insensitivity in *AtPP2CA* overexpressors and ABA hypersensitivity of loss-of-function mutants.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) L. Heynh. ecotype Columbia was used in this study unless otherwise indicated. Plant growth conditions have been described elsewhere (Nishimura et al., 2004).

### Genetic Mapping and Identification of the *AHG3* Locus

$F_2$ progeny obtained from a test cross between *ahg3-1* and L*er* were grown on MS medium containing 0.2 $\mu$M ABA for 7 d. ABA-hypersensitive seedlings without green open cotyledons were selected and grown on soil for DNA isolation and progeny. For mapping, PCR-based markers such as simple-sequence-length polymorphism and cleaved-amplified polymorphic sequence markers were used (Supplemental Table III). Isolation of genomic DNA and PCR conditions have been described previously (Hirayama et al., 1999).

### In Vitro PP2C Assay

cDNA fragments of *AHG3* and the *ahg3-1* mutant were generated by RT-PCR using primers AHG3F*Sma*I and AHG3R*Sal*I (Supplemental Table IV), and sequenced. The cDNA fragment of the *ahg3*$^{G145D}$ mutant was generated by RT-PCR using primers G145DF*Bsp*HI and AHG3R*Sal*I (Supplemental Table IV). The PCR product of *ahg3*$^{G145D}$ was digested with *Bsp*HI/*Sal*I and substituted for the *Nco*I/*Sal*I fragment of the AHG3 cDNA. The cDNAs were inserted into the *Sma*I/*Xho*I site of pGEX6P-1. Recombinant proteins (GST-AHG3, GST-ahg3, and GST-ahg3$^{G145D}$) were expressed in *Escherichia coli* DH10b cells and affinity purified on glutathione Sepharose 4B resin (Bertauche et al., 1996). Protein phosphatase activity was determined on $^{32}$P-labeled casein as substrate (Bertauche et al., 1996; Gosti et al., 1999; Mackintosh and Moorhead, 1999; Merlot et al., 2001).

### RNA Gel-Blot Analysis

Approximately 10 $\mu$g of total RNA were separated in 1.0% agarose gel containing 1.8% formaldehyde and transferred to a nylon membrane. The blot was hybridized at 60°C overnight in Church buffer (7% SDS, 0.5 M sodium phosphate buffer, pH 7.2, 10 mM EDTA), and then washed twice with 1 × SSC, 0.1% SDS for 15 min, and twice with 0.1 × SSC, 0.1% SDS for 20 min at 60°C. BAStation (Fuji Film) was used for visualization of the blot.

## Transgenic Plants

The *AHG3* and *ahg3*$^{G145D}$ cDNAs were inserted into the *Sma*I/*Xho*I site of binary vector pMSH2. The *At3g11410* (*AHG3*) genomic DNA segment was amplified by PCR using primers AHG3F*Sal*I and AHG3R*Sma*I (Supplemental Table IV) and sequenced. For the *ahg3*$^{G145D}$ mutation, the genomic fragment was generated by PCR using primers AHG3F3 and G145DR*Bsp*HI (Supplemental Table IV), digested with *Nco*I/*Bsp*HI, and substituted for the *Nco*I fragment of the genomic *AHG3* clone. Cloned fragments were inserted into binary vector pBI101. Agrobacterium GV3101 cells were transformed with these plasmids and used for infection of flowering plants by the floral-dip method (Clough and Bent, 1998).

## T-DNA Insertion Lines

T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (ABRC). Homozygous plants were identified by the kanamycin tolerance test and a PCR-based method using T-DNA left- or right-border primers (Alonso et al., 2003) and gene-specific primers.

## Generation of Double Mutants

*ahg3-1* plants were crossed with *abi1-1*, *abi2-1*, *abi3-1*, *abi4-1*, and *abi5-1* plants, and resulting $F_1$ plants were allowed to self pollinate. $F_2$ plants carrying both mutations were identified by mutant-specific cleaved-amplified polymorphic sequence markers (Supplemental Table I).

## Chlorophyll Measurement

Chlorophyll was extracted from 10-d-old plantlets in 80% acetone. Chlorophyll content was determined as described previously (Arnon, 1949).

## Measurement of Endogenous ABA Content

Samples were homogenized and extracted in 5 mL of methanol:water:acetic acid (90:9:1, v/v/v) with 2,6-di-*tert*-butyl-4-methylphenol (200 mg/L). $^{13}C_2$-ABA was added as an internal standard at the beginning of each extraction (Asami et al., 1999). After 17.5 mL of water were added, the supernatants were clarified by centrifugation and were loaded onto Oasis HLB cartridges (Waters). ABA was eluted with 1 mL methanol:water:acetic acid (90:9:1, v/v/v). An aliquot (5 mL) of each sample was loaded onto a Capcell Pac $C_{18}$ column (150 mm × 2 mm; Shiseido), and HPLC was performed using a binary solvent system of methanol and water (1:1, v/v) with 0.1% formic acid at a flow rate of 0.2 mL/min. The compounds were analyzed by tandem mass spectrometry with multiple reaction monitoring in negative-ion mode. The precursor ion (*m/z*) and product ion (*m/z*) set for each compound were as follows: 263 and 153 for the ABA standard and 265 and 153 for the $^{13}C_2$-ABA internal standard.

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